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Effect of Recombinant Human Macrophage Colony-Stimulating Factor 1 on Immunopathology of Experimental Brucellosis in Mice

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Brucella abortus injected into CBA mice replicated primarily in the spleen and liver, reaching a peak bacterial count in both organs about 7 days postinfection. The organism was eliminated from the liver but declined to a chronic phase in the spleen. The infection caused hepatosplenomegaly. An influx of macrophages into the two organs was monitored by quantitative Northern (RNA blot) analysis of the macrophage-specific marker lysozyme mRNA. Lysozyme mRNA was detectable in spleen and increased three- to fourfold during infection. In liver, lysozyme mRNA was initially undetectable, but at about the peak of infection it reached a level comparable to that in the spleen. Macrophage colony-stimulating factor 1 (CSF-1) has been reported to be elevated in the circulation of animals infected with *B. abortus* and is known to stimulate monocytopoiesis. To investigate the role of CSF-1 in pathogenesis, we studied the effect of further increasing the CSF-1 concentration by administration of recombinant human CSF-1. Since the infection is characterized by several distinct phases, recombinant human CSF-1 was administered at defined times relative to these phases. Pronounced effects were observed only when CSF-1 administration was begun during the developing acute phase. The consequences were decreased bacterial numbers in the spleen but an increase in the liver, reduced antibody generation, and increased hepatosplenomegaly. A feature of many chronic intracellular infections is immunosuppression. *B. abortus* caused a substantial diminution of responsiveness of spleen cells to T-cell mitogens, particularly concanavalin A. This action was mimicked by CSF-1 treatment of the animals prior to spleen cell isolation. The results suggest that CSF-1 plays a role in macrophage recruitment in brucellosis and that recruited macrophages contribute to the immunopathology and immunosuppression.

Brucellae are chronic infectious agents which replicate within the cytoplasm of host mononuclear phagocytes (6). Experimental murine brucellosis provides a useful model for the study of many intracellular pathogens. Four stages of infection can be identified in CBA mice: bacterial numbers increase during the first week; a bactericidal stage concurrent with the onset of cell-mediated immunity follows; by week 3, a plateau of bacterial numbers is attained; and a recovery phase begins 5 to 6 weeks postinfection. A marked hepatosplenomegaly, maximal at 2 to 3 weeks, is the major pathological consequence. Immunity to infection is believed to reside primarily in T-cell-mediated activation of macrophage antimicrobial activity (6). However, T-cell responsiveness during brucellosis is suppressed, as reflected by reduced in vitro proliferation of spleen cells from infected animals to mitogens and *Brucella* antigens (22).

The proliferation, differentiation, and mature function of mononuclear phagocytes is regulated by macrophage colony-stimulating factor 1 (CSF-1) (23). The gene for CSF-1 in humans and mice has been isolated (18, 21, 26), and the human recombinant protein (which is active in mice) is available for therapeutic evaluation. Intravenous administration of the human recombinant protein into mice causes a dose-dependent increase in the number of tissue macrophages and circulating monocytes (16), the consequences of

which are difficult to predict. In an experimental model of melanoma, CSF-1 provided substantial protection against pulmonary and extrapulmonary metastasis, despite its inability to activate macrophage tumor cytotoxicity (15). Disseminated candidiasis in mice was exacerbated by treatment with CSF-1 administered either before or after infection (13), and in an experimental model of arthritis in rats, CSF-1 increased joint inflammation in the acute phase and reactivated the disease in the chronic phase (1).

Experimental infection of mice with *Brucella abortus* has been shown to cause an elevation in circulating levels of CSF-1 (9). CSF-1 could aid the host by increasing the number of effector cells. Alternatively, the increase in macrophage numbers could aid the pathogen by providing more host target cells and/or by altering the nature of the immune response. The role of CSF-1 in the pathogenesis of intracellular infections of macrophages has been investigated to a greater extent in experimental infections with *Listeria monocytogenes*. This organism causes elevations in circulating CSF-1, blood monocyte count, and CSF-1-responsive progenitor cells in bone marrow (8). The magnitude of this response does not vary between mouse strains that differ in their ability to clear the organism, suggesting that it is not the "rate-limiting" determinant of host resistance (8). One way of testing the role of CSF-1 in intracellular infections of macrophages is to ascertain whether artificial elevation of CSF-1 concentration early in the infectious process alters the course of the disease. This article is concerned with a study of the effect of recombinant CSF-1 administration on the immunopathology of experimental brucellosis in mice.

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MATERIALS AND METHODS

Mice. CBA mice were bred by the Central Animal Breeding House, University of Queensland, and were kept under conventional but disease-free conditions. Weight-matched males, 7 to 10 weeks old, were used in all experiments.

Bacteria. *B. abortus* S19, originally from Commonwealth Serum Laboratories (Parkville, Australia), was kindly provided by C. Cheers (University of Melbourne, Melbourne, Australia). The culture was maintained by weekly subculture on *Brucella* agar (Oxoid, Basingstoke, England) containing 5% heat-inactivated horse serum (Commonwealth Serum Laboratories) and was renewed from freeze-dried stock after 20 or fewer subcultures.

Infection of mice. For the studies shown in Fig. 1 and 2, mice were infected intraperitoneally with approximately 5×10^7 CFU of *B. abortus* S19. For CSF-1 therapeutic studies, mice were injected intravenously with 100-fold fewer organisms, which gave a similar pattern of infection but reduced the variation within groups.

CSF-1 injection protocol. For most of the experiments, human CSF-1 produced by recombinant CHO cells (a gift from Genetics Institute Inc., Cambridge, Mass.) was used. CSF-1 or the phosphate-buffered saline vehicle was injected intravenously in a 0.2-ml volume in the morning on a minimum of 4 successive days. The rationale for this protocol is described by Hume et al. (16). One unit of CSF-1 activity as defined for the Genetics Institute CSF-1 and widely used for other CSFs is the amount that produces half-maximal response in the murine bone marrow assay. This is equal to 50 U as defined by Stanley (23) and as usually applied to Cetus CSF-1. Other experiments were done with recombinant human CSF-1 produced in *Escherichia coli* (a gift from Cetus Corporation, Emeryville, Calif.). This product is unglycosylated and smaller than the mammalian recombinant protein and is cleared more rapidly from the circulation. In preliminary experiments, we ascertained that a dose of 100 μ g per injection (ca. 10^5 U) caused a threefold increase in peritoneal macrophage count. Neither preparation contained detectable lipopolysaccharide activity in the *Limulus* amoebocyte lysate assay. We use both preparations routinely in studies of the growth of bone marrow-derived macrophages, a system in which CSF-1 action is blocked by lipopolysaccharide at concentrations comparable to the sensitivity of the *Limulus* amoebocyte lysate assay (see reference 13).

RNA preparation and Northern (RNA blot) analysis. Tissue was removed, immediately snap-frozen in liquid nitrogen, and stored at -20°C . The frozen tissue was placed in ice-cold lysis buffer (6 M guanidine hydrochloride, 0.2 M sodium acetate, 1 mM 2-mercaptoethanol [pH 5.2]) and homogenized immediately with an Ultra-Turrax homogenizer. Total nucleic acid was precipitated in 70% ethanol, resuspended in lysis buffer plus 10 mM EDTA, reprecipitated in 70% ethanol, and finally resuspended in 7 M urea-100 mM Tris-HCl-0.1 M EDTA-0.1% sodium dodecyl sulfate. The nucleic acid was extracted twice with an equal volume of phenol-chloroform (1:1) and once with water-saturated ether, and then 2 M potassium acetate (pH 5.0) was added to a final concentration of 0.1 M before reprecipitation in 70% ethanol. To extract the DNA and tRNA, the pellet was resuspended in 2 M LiCl on ice, then resedimented, and washed twice in 70% ethanol-0.1 M potassium acetate (pH 5.0). Finally, the pellet was resuspended in 10 mM Tris-HCl-0.1 mM EDTA (pH 8.0).

Twenty micrograms of total RNA was lyophilized and

resuspended in 4.8 μ l of water. Then, 3.2 μ l of formaldehyde, 10 μ l of formamide, and 2 μ l of $10\times$ MOPS buffer [200 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA] were added, and the sample was heated to 65°C for 10 min. Loading dye (1 μ l) containing ethidium bromide (2 mg/ml) was added, and then the RNA was separated on 1% agarose gels in $1\times$ MOPS buffer plus 3% formaldehyde. The running buffer was $1\times$ MOPS buffer. RNA was transferred by vacuum transfer (Vacublott; Pharmacia) to Hybond N nylon membranes (Amersham) as recommended by the manufacturer. Probes were labeled with a random prime labeling kit supplied by Bresatec, Adelaide, Australia. Probe hybridization conditions were as recommended by the manufacturer of Hybond N membranes (Amersham). The murine lysozyme cDNA probe was a gift from Rainer Renkawitz (Munich, Germany). RNA loading was ascertained by probing with an end-labeled oligonucleotide corresponding to the sequence of 18S rRNA. Lysozyme mRNA and rRNA were quantitated by scanning the blots with an AMBIS Radioanalyzer.

DTH. To test for delayed-type hypersensitivity (DTH), mice were challenged by injection of 10 μ g of *B. abortus* soluble antigen into the hind footpads. The antigen was prepared by the method of Berman et al. (4) except that it was derived from *B. abortus* S19 cells propagated on *Brucella* agar with horse serum. Footpad thickness, measured with a dial micrometer (Mitutoyo, Tokyo, Japan), was determined immediately before and 24 h after challenge, and footpad swelling was calculated by subtracting the initial value from the postchallenge value.

Bacterial counts. Suspensions of infected tissues (spleen, liver) from infected mice were prepared in a peristaltic homogenizer (Seward Stomacher, London, England), and serial 10-fold dilutions were plated in triplicate on Columbia agar (Oxoid). Colonies were counted after 72 h of incubation at 37°C .

Peritoneal cell counts. Peritoneal cells were obtained by lavage with heparinized Hanks balanced salt solution and enumerated in a hemacytometer.

Antibody titration. Mice anesthetized with ketamine-xylazine were bled from the heart into heparinized syringes. Plasma was obtained by centrifugation and titrated against whole *B. abortus* cells by the microtiter spin agglutination assay (27). *B. abortus* bacterial suspension (Commonwealth Serum Laboratories) was added in equal volumes to microtiter wells containing plasma serially diluted in phosphate-buffered saline. Following 1 h of incubation at room temperature, the microtiter plate was centrifuged at $1,000 \times g$ for 5 min. Before being read, the plate was inclined at an angle of approximately 45° for 5 min. A positive test was characterized by a distinct button of agglutinated cells, whereas in a negative test the cell pellet streamed. Results are expressed as reciprocal titers.

Statistical analysis. The statistical significance of differences between control and CSF-1-treated groups was assessed by the Mann-Whitney rank order test. The standard error of the mean is recorded on tables and figures to give an indication of variance, but the data, particularly for bacterial counts, were clearly skewed towards lower values and were not normally distributed.

Tissue culture medium. RPMI 1640 (Commonwealth Serum Laboratories) was supplemented with 10 mM sodium bicarbonate, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, and antibiotics (streptomycin [100 mg/liter] and penicillin [10^5 U/liter]).

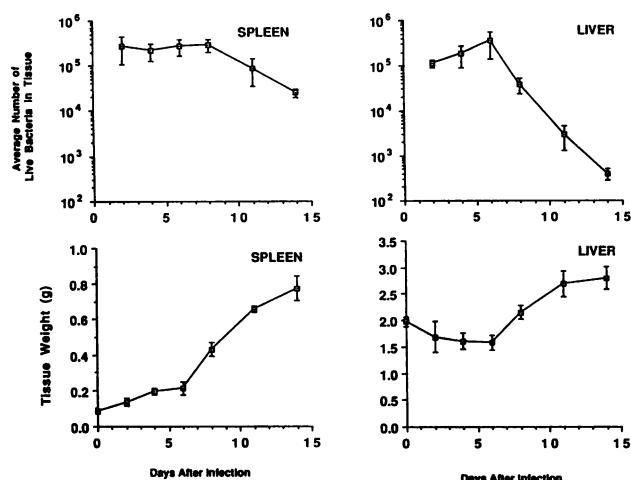


FIG. 1. Time course of infection and changes in spleen and liver weights in *Brucella*-infected mice. Means are the average \pm standard error (SE) for four animals from two separate experiments.

Lymphocyte proliferation assays. Single-cell suspensions were prepared from spleens excised from untreated controls or from mice 5 days after infection or commencement of CSF-1 administration, by teasing tissue through stainless steel mesh into cold RPMI. Erythrocytes were removed by ammonium chloride lysis. Cells (2×10^5) were added to each U-bottomed microtiter well in 200 μ l of RPMI with 2% normal mouse serum plus dilutions of phytohemagglutinin (PHA; Sigma, St. Louis, Mo.) or concanavalin A (ConA; Calbiochem, La Jolla, Calif.). After 24 h of incubation, each culture was pulsed with 37 kBq of [*methyl*- 3 H]thymidine (Amersham, Buckinghamshire, England) for 16 h. Cells were then harvested onto glass fiber filters, and incorporation of the radiolabel was quantitated by liquid scintillation spectroscopy. All determinations were done in triplicate.

RESULTS

Bacterial infection time course. Figure 1 shows a time course of infection in animals infected with *B. abortus*. The patterns observed in the two major organs affected, the liver and spleen, were somewhat different. Peak bacterial numbers were reached very rapidly in the spleen, within 2 days of infection, and remained stable until day 8, when they started to decline rapidly. In the liver, bacterial numbers rose more slowly, reaching a peak on day 6 at a higher level than observed in the spleen, and then declined much more rapidly, so that by day 14 the numbers approached the limits of detection. These data are essentially compatible with those of Cheers (6).

Hepatosplenomegaly. Both the spleens and the livers of infected animals increased in weight during infection (Fig. 1). Spleen weight had increased twofold by day 6 and increased a further fourfold by day 14. Liver weight was stable up until day 6 but increased by approximately 50% by day 14. Note that the largest increases in weight in both organs appear to correlate with the resolution of the infection (Fig. 1).

Northern analysis of mRNA expression in infected organs. In order to gain some insight into the role of macrophages in hepatosplenomegaly, we isolated RNA from the livers and spleens of *Brucella*-infected animals at various times postinfection and investigated the expression of macrophage-

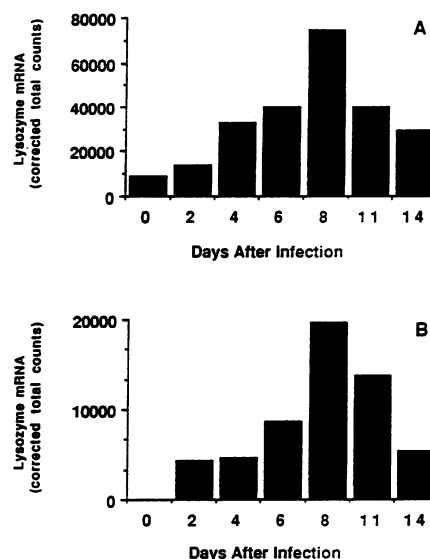


FIG. 2. Effect of *Brucella* infection on expression of lysozyme mRNA in the liver and spleen. Northern blotting and hybridization of tissue RNA prepared from animals on the day indicated were performed as described in Materials and Methods. The radiolabel (counts per minute) associated with the lysozyme-specific band was quantitated with an AMBIS Radioanalyzer and is corrected relative to the labeling intensity of the 18S RNA band on the same sample. Results are the average of duplicate spleens (A) and livers (B) processed from separate infected animals.

specific mRNAs. In granulocytes and other cell types, lysozyme is stored in cytoplasmic granules, and the mRNA is not abundant. By contrast, macrophages produce the protein constitutively and secrete it into the extracellular medium. Lysozyme mRNA has been reported to be very abundant in murine macrophages but not in granulocytes (5, 10). We have confirmed that lysozyme mRNA is expressed in primary mouse macrophages and macrophagelike tumor cell lines at very high levels, comparable to β -actin (data not shown). Because of this abundance, lysozyme mRNA can be detected in total RNA from whole organs even though macrophages represent a relatively small percentage of the total cell number.

Lysozyme mRNA was detectable in control spleen, increased substantially by day 2 to day 4 postinfection, and reached an apparent peak by days 6 to 8 (Fig. 2A). In control liver, no lysozyme mRNA could be detected, but the level approached that of infected spleen by days 6 to 8 postinfection. The blots used for lysozyme analysis were reprobbed with cDNA encoding *c-fms*, the receptor for CSF-1, and urokinase plasminogen activator, a CSF-1-inducible gene (13). No band was detectable in control liver or spleen, but bands of the expected sizes were obtained with liver and spleen samples taken on days 6, 8, 11, and 14 (data not shown). The signal was below the limits of accuracy of the AMBIS Radioanalyzer and was not quantified. Nevertheless, the observation supported the conclusion that substantial macrophage infiltration of both organs occurred during the infection.

Effect of treatment with CSF-1 on *Brucella* infection. Because macrophage infiltration of the spleen and liver correlated with reduced bacterial count, and because CSF-1 administration can cause macrophage infiltration into both sites (16), animals were treated with recombinant CSF-1 to

TABLE 1. Effect of CSF-1 pretreatment on *Brucella* infection^a

Group	Liver wt (mg)	Spleen wt (mg)	DTH (footpad swelling, μ m)	Agglutinating antibody titer	<i>B. abortus</i> (10^6 CFU/spleen)
Control	1,504 \pm 54	128 \pm 9	11 \pm 6	11.6 \pm 1.6	3.9 \pm 1.4
CSF-1 treated	1,561 \pm 64	112 \pm 8	45 \pm 14	5.0 \pm 0.5	2.4 \pm 0.7

^a Animals were injected intravenously with recombinant human CSF-1 on each of the 4 days preceding intravenous infection with 5×10^5 *B. abortus* organisms. Results are expressed as the mean \pm SE ($n = 6$). The differences between control and treated animals are not significant ($P > 0.05$).

see whether it would hasten the resolution of the disease. Since the time course of experimental brucellosis exhibits a series of well-marked phases, CSF-1 was administered at several defined times in relation to the initiation of infection. When CSF-1 was given to mice on 4 consecutive days (days -4, -3, -2, and -1) prior to infection (on day 0), there was no significant effect on total body weight, liver and spleen weight, DTH reaction, antibody titer, or bacterial count in the spleen. Measurements were made on day 7, during the acute phase, and the results are shown in Table 1. Slight decreases in the mean values for bacterial load and antibody were not significant ($P > 0.05$) by the Mann-Whitney method. The normal mouse spleen weight (80 ± 8 mg [standard deviation]) was increased by the infection but was not further influenced by CSF-1 in this experiment. Thus, CSF-1 had no definable prophylactic activity. This result implies that the effect of CSF-1 is reversible, in that the increased spleen weight observed when animals are killed on the day after the last CSF-1 injection (see reference 16 and Table 2) was no longer evident 7 days later.

Effect of CSF-1 administration at time of infection. Treatment with CSF-1 commencing at the time of infection (day 0) did alter several parameters. Again, the CSF-1 was administered on 4 consecutive days, and the results on day 7 for three separate experiments are shown in Table 2. The efficacy of the CSF-1 was demonstrated by a three- to fourfold increase in peritoneal cell number, as observed previously in uninfected animals (16) and confirmed in uninfected controls assayed in parallel (not shown). CSF-1 also increased spleen mass above the approximate doubling caused by *Brucella* infection itself. The magnitude of the increase was similar to that reported in an earlier study of uninfected animals (16). Neither *Brucella* infection nor CSF-1 alone caused any change in liver weight at this time

point compared with that in three untreated animals processed at the same time (data not shown, but see also day 6/day 8 time point in Fig. 1). They did cause a 15 to 20% increase in combination.

The bacterial counts in the two organs changed in opposite directions. The count in the spleen decreased significantly in one of three experiments and was unaffected in the others. Conversely, the bacterial count in the liver was increased five- to sixfold by CSF-1. At this early stage in infection, DTH and antibody responses were still quite low. The DTH response was unchanged by CSF-1, but the agglutinating antibody titer was reduced twofold. Note that the third experiment in Table 2 was performed with the bacterial CSF-1 from Cetus with essentially the same result. In this third experiment, we also looked at the lungs, another site for macrophage-mediated clearance of intravenous inocula. There were far fewer organisms in the lungs than in liver and spleen. Lung weight and bacterial load of *Brucella* organisms were unaffected by CSF-1. There was also no change in the number of endogenous floras obtained on the assay plates.

Effect of CSF-1 treatment during chronic phases. As shown in Fig. 1, the bacterial count in the spleen and liver was much reduced by day 14 postinfection. This decline in the number of organisms was correlated with potent antibody and DTH responses, which were sustained for an extended period (Table 3). It seemed inherently unlikely, given the elevation in CSF-1 mRNA in liver and spleen and the reported increase in biological activity in the circulation (9), that addition of more CSF-1 would alter any parameters of infection or pathogenesis. However, the presence of CSF-1 does not necessarily mean it is saturating for mononuclear phagocyte receptors. If it was not, we believed that administration late in infection could reactivate bacterial replication by providing naive target cells.

TABLE 2. Effect of concurrent CSF-1 treatment on *Brucella* infection^a

Group and expt no.	Liver wt (mg)	Spleen wt (mg)	Lung wt (mg)	DTH (footpad swelling, μ m)	Antibody titer	Peritoneal macrophages (10^6)	<i>B. abortus</i> counts			Total flora (10^4 CFU/lung)
							10^6 CFU/spleen	10^5 CFU/liver	10^4 CFU/lung	
Control										
I	1,424 \pm 110	145 \pm 8	122 \pm 6	64 \pm 18	90 \pm 12	1.4 \pm 0.3	7.7 \pm 1.1			
II	1,503 \pm 38	173 \pm 9		15 \pm 6	37 \pm 5	2.1 \pm 0.5	11.0 \pm 3.7	6.4 \pm 0.9		
III	1,444 \pm 32	173 \pm 12		35 \pm 7			30.0 \pm 5	15.0 \pm 3.0	14.0 \pm 12.0	18 \pm 7
CSF-1 treated										
I	1,765 \pm 64 ^b	226 \pm 11 ^c	133 \pm 7	41 \pm 14	35 \pm 6 ^b	5.1 \pm 1.6 ^b	3.6 \pm 0.6 ^c			
II	1,833 \pm 22 ^c	254 \pm 10 ^c		24 \pm 11	17 \pm 2 ^c	7.3 \pm 1.2 ^c	8.7 \pm 2.7	24.9 \pm 7.4 ^c		
III	1,511 \pm 46	216 \pm 12 ^b		42 \pm 13			32 \pm 6	71.0 \pm 8.0 ^c	2.3 \pm 0.8	11 \pm 4

^a CSF-1 treatment was begun on the same day as injection of 5×10^5 *B. abortus* organisms and continued daily for 4 days. Animals were killed on day 7 postinfection. Results are expressed as the mean \pm SE ($n = 10$) and derive from three independent experiments (I, II, and III). CSF-1 from the Genetics Institute was used for experiments I and II, and CSF-1 from Cetus was used for experiment III.

^b $P < 0.05$ versus control.

^c $P < 0.01$ versus control.

TABLE 3. Effect of CSF-1 treatment during chronic phase of *Brucella* infection^a

Group and expt no.	Liver wt (mg)	Spleen wt (mg)	DTH (footpad swelling, μ m)	Antibody titer	Peritoneal macrophages (10^6)	<i>B. abortus</i> (10^4 CFU/spleen)
Control						
I	1,534 \pm 45	280 \pm 36	222 \pm 38	1,280 \pm 143	1.3 \pm 0.6	6.7 \pm 2.4
II	1,674 \pm 75	175 \pm 12	152 \pm 35	1,061 \pm 189		2.3 \pm 1.0
CSF-1 treated						
I	1,619 \pm 52	305 \pm 18	135 \pm 46	1,052 \pm 71	1.8 \pm 0.6	2.6 \pm 1.4
II	1,673 \pm 69	217 \pm 29	219 \pm 25	640 \pm 134		1.8 \pm 1.4

^a Treatment with CSF-1 was begun on either day 35 (experiment I) or day 46 (experiment II) postinfection. In experiment I, treatment was given for 4 days, and the animals were killed on the seventh day. In experiment II, treatment was given for 6 days, and the animals were killed on the seventh day. Results are means \pm SE ($n = 10$). Differences between control and CSF-1-treated groups are not significant ($P > 0.05$).

In the first experiment, CSF-1 was given on 4 consecutive days beginning on day 35. In the second experiment, an attempt was made to amplify the small effects by treating more often (on 6 days) and starting later (day 46), when the bacterial count had fallen further. There were no convincing changes observed in response to CSF-1 in spleen mass, DTH, or antibody titer, all of which were already greatly elevated. The bacterial count in the spleen was also not significantly reduced. By this time, bacteria in the liver were undetectable. Most importantly, there was no detectable effect of CSF-1 on peritoneal cell numbers, suggesting that the animals were no longer responsive to additional exogenous ligand.

Time course of suppression of lymphocyte proliferation by infection. The data thus far suggested that elevation of plasma CSF-1 was not sufficient to hasten the clearance of bacteria from the spleen and liver. In fact, they are more consistent with a role in recruitment of target cells and the generation of hepatosplenomegaly. Accordingly, CSF-1 might play a role in another well-defined index of the immunopathology of brucellosis. Immunosuppression is a common feature of chronic intracellular infections, which manifests in a decreased responsiveness to T-cell mitogens. The ability of macrophages from *Brucella*-infected mice to inhibit mitogen responses of splenic T lymphocytes has been reported previously (22). Since there is abundant evidence that different T-cell mitogens affect different cell populations (17), we looked at the different effects of *B. abortus* on spleen cell responsiveness to two different mitogens, ConA and PHA, at a range of concentrations.

Responsiveness to all concentrations of ConA was severely diminished in spleen cells from animals assayed 10 and 20 days after *Brucella* infection but was restored, with low-dose enhancement, by day 30 postinfection (Fig. 3). Inhibition of proliferation in response to PHA was not as severe. There were small changes in the dose-response curves at days 5 and 10 postinfection, but by day 20 the response was greater than in control spleen cells (Fig. 4). Since *B. abortus* alone was capable of producing substantial immunosuppression, there was little point in assaying the effect of CSF-1 therapy. We therefore asked whether CSF-1 alone could produce a similar suppression of mitogen responses. Figures 3 and 4 (day 5) show that the alteration of spleen cell proliferative responses to ConA and PHA caused by treatment of mice with CSF-1 was even more pronounced than seen during *Brucella* infection. In the microcultures, the increased number of macrophages in the spleens of CSF-1-treated animals was visibly obvious, with numerous cells attached to the sides of the U-shaped wells (not shown).

Both ConA and PHA responses were greatly inhibited. As seen in *Brucella*-infected spleens, the optimal concentration of ConA was also reduced (from 5 to 1 μ g/ml).

DISCUSSION

The results address some aspects of the role of CSF-1 in the pathogenesis of a chronic intracellular infection, exploring in the process any possibility of a therapeutic application. The time course of *Brucella* infection was shown to differ between the two major involved organs (Fig. 1). In the spleen, the infection peaked early and declined to a plateau of about

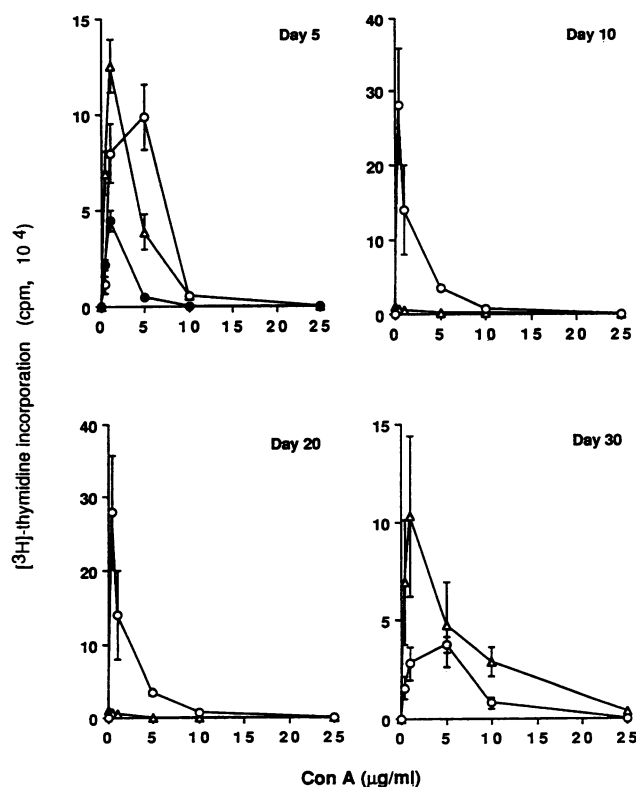


FIG. 3. Dose-response curves for ConA-induced proliferation at various times postinfection. Spleen cells from infected mice (Δ), uninfected control mice (\circ), and mice treated for 4 days with CSF-1 (\bullet ; assayed in parallel with day 5 infection samples) were tested. Points and bars represent means \pm SE ($n = 4$).

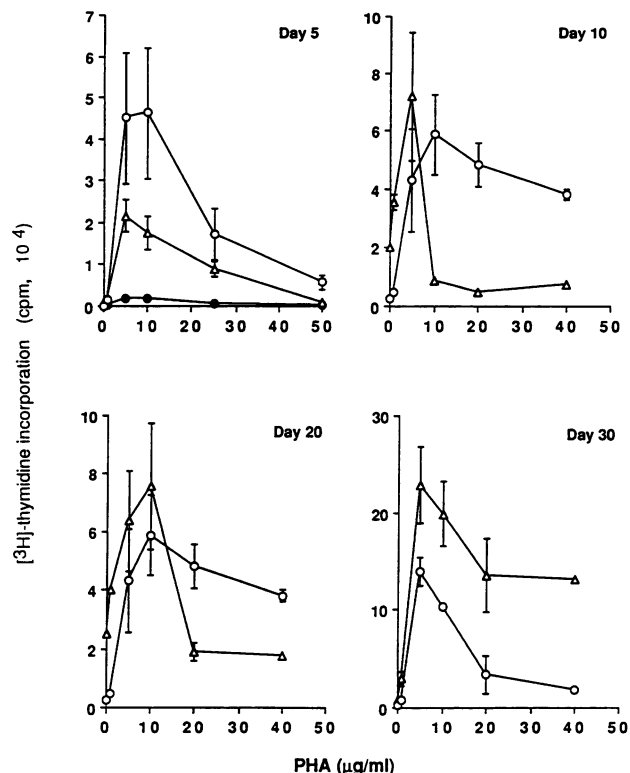


FIG. 4. Dose-response curves for PHA-induced proliferation at various times postinfection. Spleen cells from infected mice (Δ), uninfected control mice (\circ), and mice treated for 4 days with CSF-1 (\bullet ; assayed in parallel with day 5 infection samples) were tested. Points and bars represent means \pm SE ($n = 4$).

5×10^4 organisms per spleen. This low level of infection can be sustained for a very long time, as documented by others (6) and as evidenced by the data in Table 3. By contrast, the liver reached a peak of infection later, but the number of organisms dropped much more rapidly. Essentially, the infection of the liver resolved completely. In both sites, there was clear evidence of substantial macrophage infiltration, as evidenced by expression of the macrophage-specific marker lysozyme mRNA (Fig. 2). Increased lysozyme mRNA expression was correlated with a rapid increase in weight in both the spleen and liver, suggesting that macrophage influx contributes to hepatosplenomegaly.

The time course of appearance of CSF-1 in the circulation of infected animals (9) parallels the major increase in hepatosplenomegaly commencing at about day 7. The ability of exogenous CSF-1 to increase macrophage numbers in the peritoneal cavity, liver, and spleen and to increase spleen weight has been documented before (16). Liver weight was not previously measured. CSF-1 alone did not cause increased liver weight (data not shown), but it accelerated the increase brought about by *Brucella* infection (Table 2). A possible interpretation is that the *Brucella* organisms provide a chemoattractant to direct the monocytes recruited by CSF-1 treatment to the site of infection. Supporting this concept, peritoneal cell macrophage numbers were not affected by intravenous infection with *B. abortus* (the uninfected control for Table 1 is about 1×10^6 to 2×10^6 per animal), but intraperitoneal infection with *B. abortus* can cause a 10-fold increase in peritoneal macrophage numbers (data not shown).

Only when CSF-1 was administered during the developing acute phase were the consequences marked; administration before infection or during the chronic phase had much less effect on any variable measured. The failure of pretreatment to influence bacterial growth or immunity suggests that CSF-1-stimulated macrophages are not cytotoxic towards *B. abortus*. Similarly, Cheers et al. (7) found that CSF-1 treatment of macrophages in vitro increased the uptake of *L. monocytogenes* but did not alter cytotoxic activity. When CSF-1 was given to mice commencing at the time of infection, the bacterial count in the spleen was reduced slightly in spite of the increase in spleen weight. Since an influx of macrophages contributes significantly to splenomegaly, a reduced or even an unchanged count would imply a considerable reduction in the number of bacteria per macrophage if all the organisms were intracellular. However, we are not able to confirm this proposal, and indeed, a possible mechanism for chronic infection of the spleen might involve *Brucella* survival in a nonmacrophage compartment. We have not monitored bacterial numbers for a more extended period following CSF-1 therapy to see whether the rate of clearance of organisms was accelerated subsequently.

In the liver, the bacterial count was increased substantially by CSF-1 treatment (Table 2). The magnitude of the change was similar to the approximate change in macrophage numbers measured in the peritoneum of the infected animals. Hence, the simplest explanation would be that CSF-1 increased the number of target cells without greatly altering their function or the rate of replication of *B. abortus* in any particular cell. If so, the question is why the same thing does not happen in the spleen. Apart from the possibility that the organisms in the spleen are not actually intracellular (see above), there could also be differences in macrophage function between the two organs. One difference is that CSF-1 mRNA is already readily detectable in the uninfected spleen but not in the liver (2a). If CSF-1-stimulated macrophages are the optimal host cells for the organism, this might explain why bacterial numbers are twofold higher in spleen than in liver by 2 days postinfection (Fig. 1) despite the fact that the liver is by far the most important organ in terms of macrophage-mediated clearance activities (14). For example, in the clearance of CSF-1 itself, 89% of radiolabeled CSF-1 is cleared by endocytosis in the liver, whereas only 6% is cleared by the spleen (3).

The data in Table 3 imply that the effects of *Brucella* infection and exogenous CSF-1 treatment on liver and spleen weight are not additive. There was no evidence that CSF-1 reactivated bacterial replication once it had entered a chronic phase. From a therapeutic viewpoint, this is not a trivial observation. The failure of CSF-1 to induce increased peritoneal cell numbers in the chronically infected animals suggested that they were resistant to the treatment. One explanation is that the CSF-1 concentration in the circulation is already very high, and the receptor pool is saturated. An alternative is that the macrophage pool is very greatly enlarged and the injected CSF-1 is much more rapidly cleared by receptor-mediated endocytosis (3). Finally, it could be that biological effectors generated by either the organism or the immune system block the action of CSF-1. We have no evidence to distinguish among these alternatives.

The reduced antibody titers seen in acutely infected animals (Table 2) suggest a decrease in immune function as a result of CSF-1 treatment. A possible mechanism for this would be the immunosuppression brought about by increased macrophage numbers. These cells are known to

downregulate immune responses under some conditions, prostaglandin release being a commonly implicated mechanism (11). The slight reduction in bacterial numbers in the spleens of CSF-1-treated mice is unlikely to influence antibody production by limiting the supply of bacterial antigens, particularly as these are known to be highly immunogenic. The protective role of antibody in murine brucellosis has been repeatedly demonstrated (19, 20), and this is probably due largely to enhancement of phagocytic capacity by opsonization (2). CSF-1 increases expression of the Fc receptor type III subclass on macrophages in vitro, which could potentially enhance the uptake of opsonized bacteria (28). However, this would bring little therapeutic benefit if the agent simultaneously reduced antibody production. As shown in Fig. 3, CSF-1 injection alone can mimic the ability of *Brucella* infection to suppress the responsiveness of spleen cells to T-cell mitogens. This finding in vivo parallels a report that CSF-1-stimulated peritoneal macrophages added to spleen cells markedly inhibited the response to T-cell mitogens (25). In addition, the course of suppression of lymphocyte proliferation by infection correlates closely with elevation of serum levels of colony-stimulating factors in infected mice: macrophage colony-stimulating activity was elevated at 7, 14, and 21 days postinfection, returning to normal by day 28 (9, 22). This evidence suggests that endogenous CSF-1, which is produced in response to *Brucella* infection, could contribute to the suppression of mitogenic responses. A possible mechanism is suggested by the discovery that CSF-1 increases production of a specific inhibitor of interleukin-1 action by murine macrophages (24).

In this context, the selectivity of inhibition of mitogenesis in *Brucella* infection towards ConA-responsive spleen cells is of interest. Hume and Weidemann (17) reviewed the evidence that ConA and PHA stimulate different, though overlapping, T-cell populations. Cells responding to low doses of ConA correlated with CD4-positive helper T-cell activity, whereas depletion of CD8-positive cells reduced PHA responsiveness. Conversely, depletion of Fc receptor or major histocompatibility complex class II-positive cells from splenocytes left a residual population that responded only to PHA. Without performing specific studies with T-cell surface markers, we do not wish to overinterpret the present results except to say that they are consistent with a selective effect of *Brucella* infection on subpopulations of T cells in the spleen. The effect of CSF-1 was rather less specific in that both PHA and ConA responses were greatly reduced (Fig. 3 and 4). Spleen cells from CSF-1-treated animals also fail to stimulate or respond in a one-way primary mixed lymphocyte reaction to allogeneic cells (13a). Of course, the situations in a *Brucella*-infected and a CSF-1-treated spleen are not comparable. In the infected spleen, the increase in macrophage content must be accompanied by an influx of other cell types, so that the relative percentage of macrophages may not change greatly (as evidenced by the data in Fig. 2).

The results suggest that macrophage numbers and the production of CSF-1 are not limiting in the control of infection with an intracellular pathogen such as *B. abortus* in the sense that increasing them produces no beneficial effect. This is in conceptual agreement with data from experimental *Listeria* infection, in which genetic variation in resistance between mouse strains could not be correlated with CSF-1 production or action (8). In fact, it appears that the ability of *B. abortus* and similar agents to induce CSF-1 is a positive virulence determinant, leading to an increase in the number of susceptible target cells (at least in some organs) and

suppressing the development of T-cell-mediated immune responses. Given these results, it will be of particular interest to ascertain whether CSF-1 influences the formation of granulomas, the most common manifestation of chronic intracellular infections.

In other systems, experimental candidiasis and streptococcal arthritis, CSF-1 administration aggravates the pathology (1, 13). In each case, there is other substantive evidence that macrophages are responsible for the pathology and that local CSF-1 production occurs. If increased CSF-1 production does play a role in pathogenesis, therapeutic applications could be envisaged for neutralizing antibodies against CSF-1 (12). However, the effect of such antibodies would be very difficult to control and to interpret because of the role of CSF-1 in the production and continued viability of macrophages in the steady state (3, 23) and the probable multiple roles of the macrophage in infection, as a host cell for bacterial replication and an effector cell in bacterial elimination and pathogenesis.

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